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Effects of temperature on the susceptibility of insect cells to infection by baculoviruses

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Abstract. Three insect cell lines were tested for susceptibility to baculovirus infection by use of a typical endpoint assay procedure. Cell lines from *Spodoptera frugiperda* (IPLB-Sf21AE), *Lymantria dispar* (IPLB-LdEIta), and *Heliothis virescens* (IPLB-HvE6s) in 96-well tissue culture plates were each infected with dilutions of extra cellular virus suspensions of the *Autographa californica* nucleopolyhedrovirus (AcMNPV). In addition, the *L. dispar* and *H. virescens* cells were also infected with *L. dispar* nucleopolyhedrovirus, and *Helicoverpa zea* nucleopolyhedrovirus, respectively. Each cell/virus combination was incubated at three temperatures: 22, 27 and 32 °C and wells were scored for positive infection (presence of occlusion bodies in cell nuclei) at 2 to 4 day intervals for up to 4 weeks. The resulting data were analyzed by the Spearman-Kärber method, providing virus titers for each combination of virus, cell line, and temperature. The results were categorized by accuracy (assuming the highest titer achieved was the most accurate) and by rapidity of maximum titer. AcMNPV reached the highest titer in each line at 22 °C although equivalent titers were reached with both AcMNPV and HzSNPV in the HvE6a line at all three temperatures. This line actually reported about 100-fold less AcMNPV than the other two lines with the same virus sample. Alternatively, the Sf21AE and LdEIta lines reached 10-fold higher titers at the lowest temperature as compared with the higher temperatures, although also at a slower rate.

Key words: AcMNPV, Cell cultures, *Heliothis virescens* Nucleopolyhedrovirus, HzSNPV, LdMNPV, *Lymantria dispar*, *Spodoptera frugiperda*, Temperature effects, Virus infectivity

Introduction

Insect cell cultures are useful tools in the study and production of viruses, especially baculoviruses in the subgroup nucleopolyhedrovirus (NPV). To provide adequate levels of infectious material during the inoculation of cultures, the virus inocula need to be accurately titered. Typically, this is accomplished by plaque assay or endpoint assay [1]. In the former, virus dilutions are inoculated onto an even lawn of cells, overlaid with a solid, semisolid or viscous nutrient solution to reduce spread of the progeny virus and incubated until foci of infection can spread to neighboring cells to form an area of infection (plaque). This procedure requires cells that attach firmly enough so that the manipulation of the cultures through the addition or removal of virus and subsequent overlaying with the nutrients does not damage or otherwise disturb the cells. Additionally, the cells must remain susceptible for a sufficient period of time so that the initially infected cell can produce progeny virus that create the secondary infections. When carefully performed, the resulting number of plaques can be an accurate representation of the number of virions in the original suspension. However, reproducibility of this technique can be difficult to achieve. The use of solid overlays such as agarose requires careful temperature control during the course of setting up the infections to keep the material liquid yet not cause a heat shock effect or death of the cells. Many insect cell lines attach poorly or not at all which can hinder the use of plaque assays.

Endpoint assays are titering methods in which multiple cultures are inoculated with virus dilutions, usually in small volumes in multiwell plates, and then examined after sufficient time has elapsed so that cytopathology can be seen. These do not have as many constraints as plaque assays and are generally more easily reproduced. This technique does not rely on cell attachment, virus spread does not need to be restricted to neighboring cells thereby eliminating the need for a special substrate, and the typical methods do not require any manipulation of the cells beyond that used for normal subcultivation. Unlike plaques assays, however, the results are not a direct measure of the number of virus particles, instead relying on the probability of cells in the inoculated cultures becoming infected.

In previous studies, colleagues and I [3, 4, 8] have

typically used endpoint assays held at room temperatures (~22 °C) instead of the optimal temperatures for cell growth (25–28 °C) under the belief that cells remain viable for a longer period of time, which in turn allows the infection to proceed for a longer period and results in greater accuracy in the titers obtained. While we had intuitive feelings that this was true, we did not have empirical evidence. In this paper, I examine the endpoint titers determined by cells at three incubation temperatures, 22, 27, and 32 °C. The same virus dilutions of *Autographa californica* NPV (AcMNPV) were used on each of three permissive cell lines and two of the lines were also infected by other NPVs. The results indicate that the highest titers were achieved on all three lines and each virus at 22 °C although the time needed to reach the final titer varied from 10 days (*Lymantria dispar* cells) to 3 weeks (*Heliothis virescens* cells). The results also suggest different rates at which the maximum titers were obtained in each line at the various temperatures.

Materials

A. Equipment
   1. Ambi-Hi-Low, model 3550 incubator[1]
   2. Hotpack, model 535 incubator[2]
   3. Model No. 100 incubator[3]

B. Supplies
   1. 96-well tissue culture plates, 3595[4]
   2. Ex-Cell 400 medium, 14400-500M[5]
   3. Fetal bovine serum (FBS, Rehatuin®), 1020-01[6]
   4. TC-100 medium, 11600-012[7]

Procedures

Cell Lines and Media. Cell lines used in these studies included IPLB-Sf21AE from *Spodoptera frugiperda* [10], IPLB-LdEIta from *Lymantria dispar* [7] (both grown on Ex-Cell 400 medium) and IPLB-HvE6a from *Heliothis virescens* [9] grown on modified TC-100 containing 9% (v/v) FBS [6].

Virus. Virus inocula of clones of AcMNPV (AcMNPV-pxh), LdMNPV (LdMNPV-a624), and HzSNPV were as described previously [5].

Cells of each line were counted *in situ* using a Nikon TMS inverted microscope fitted with a calibrated reticulated eyepiece. After counting, cells were suspended by flushing with medium from a transfer pipet and diluted in their respective medium to 1×10^5 cells/ml. Cells (5,000 in 0.15 ml) were distributed into each well of a 96-well plate. The virus samples were diluted through 10-fold serial dilutions in modified TC-100 to 10^{-9} of the original. Aliquots (50 µl) of each dilution were inoculated onto each of 12 wells of the respective cell lines. The plates were sealed in airtight plastic boxes with moistened towels to maintain humidity and placed in incubators at 22, 27 or 32 °C. Cells were examined for cytopathic effect (the presence of occlusion bodies in the nuclei) at 2 to 4 day intervals up to 4 weeks post infection. The resulting data were analyzed by the Spearman-Kärber method [2].

Results

The resulting titers obtained with the various temperature/cell/virus combinations are presented in Figure 1. The comparisons of AcMNPV in the three cell lines (Figures 1A–C) revealed the virus showed the highest titer in each line when incubated at 22 °C. This supports the original hypothesis that this temperature provided the most accuracy for titration. However, as would be expected, the time required to reach the maximum titer was slower at this temperature (Table 1). The LdEIta line reach the maximum titer earliest at 22 °C at 2 weeks (Figure 1B), while Sf21 and HvE6a each required 3 weeks at this temperature (Figures 1A and 1C, respectively). Alternatively, the highest titer was reached in the Sf21 line at only 5-days post infection at 32 °C although this was 26 fold lower than that obtained at 22 °C. Interestingly, the HvE6a line showed little difference between the three experimental temperatures (the titer at 22 °C was only 20% greater than that at 32 °C and within the standard error for the experiments). However, the maximum titer obtained with this line was only about 8% of that obtained with the other two lines (notice the different y-axis scale used for Figure 1C compared to 1A and 1B), suggesting this line was less susceptible to AcMNPV than the other cells used in this study.

Similar results were obtained with the other two viruses in their permissive cell lines. The maximum titer was obtained with LdMNPV in LdEIta at 22 °C 3 weeks after infection (Figure 1D) and the maximum titer with HzSNPV in the HvE6a cells was similar at all three temperatures (Figure 1E). Again, the maximum titer of virus on LdEIta cells at 32∞C was obtained one-week post infection but was 300-fold less than the maximum obtained at 22∞C and the titers of HzSNPV in HvE6a at the three lines were similar at each temperature although obtained at progressively later times as the temperature decreased.

Discussion

Optimum titer vs. speed

The results in this study confirmed the intuition that the highest final titer could be obtained if the cells were held at a lower temperature. The disadvantage

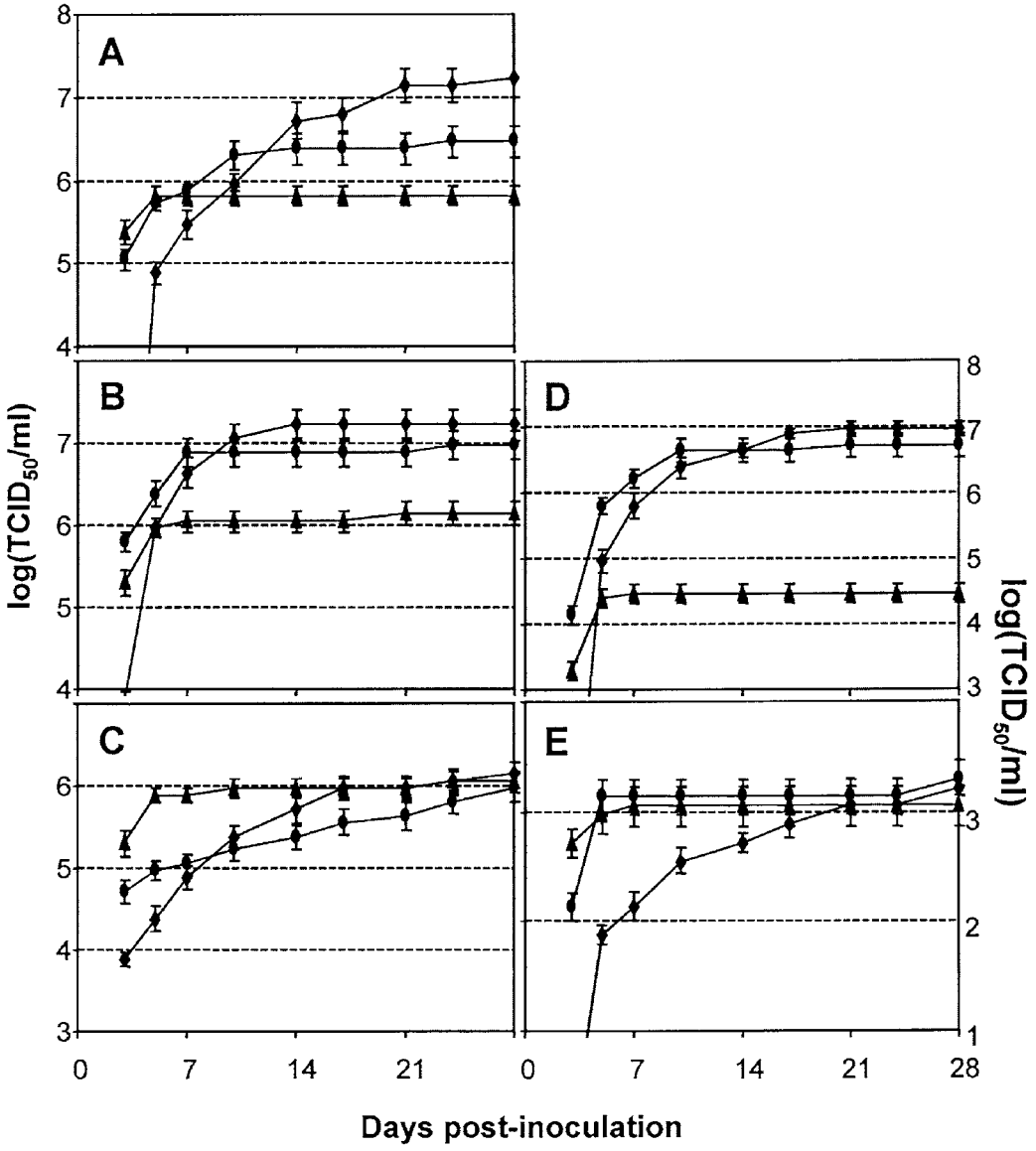

Figure 1. Endpoint titers ($TCID_{50}$/ml) on a logarithmic scale at various times post-inoculation. Each point is the titer as determined by Spearman-Kärber method; error bars are 1 standard error determined by the same technique. A. AcMNPV on Sf21 cells; B. AcMNPV on LdEIta cells; C. AcMNPV on HvE6a cells; D. LdMNPV on LdEIta cells; E. HzSNPV on HvE6a cells. Data points: ◆ 22 °C; ● 27 °C; and ▲ 32 °C on each graph.

of this technique over the use of higher temperatures is the time involved, up to two weeks longer for the final results (Table 1). If the titration is being performed on virus intended for use as a stock suspension, the greater accuracy will probably be worth the slower time for obtaining the results. However, frequently in a production situation, virus titers would be needed quickly and in these situations, it may be worth the loss in accuracy to obtain the results more quickly.

Cell vs. virus

The results obtained with the different viruses suggest that temperature variations are mostly related to the cell. Replication in both Sf21 and LdEIta cells appear to be inhibited at the highest temperature. Based on the appearance of the cells, this may be explained by the general health of the cells. At 32 °C, both Sf21 and LdEIta become granular and form aggregates within a few days, both an indication of poor health. The HvE6a cells at this same temperature appear healthy for at least 3 weeks, suggesting they may remain susceptible to infection for a longer period, which would explain the more consistent final titers in this line at the three temperatures. Each of the cell lines used in this study grows optimally in the 26–28 °C range.

As an aside to this study, the normal insect habitat could be used to explain the differences between the different cell lines. *L. dispar* is a forest canopy insect in temperate climates and would typically be exposed

Table 1. Length of assay time to reach maximum titers at each temperature.

Cell line	Virus	Temperature (°C)	Time (days) to maximum titer	Maximum titer ($TCID_{50}$/ml)
IPLB-Sf21AE	AcMNPV	22	28	1.65×10^7
		27	24	2.94×10^6
		32	5	6.32×10^5
IPLB-LdEIta	AcMNPV	22	14	1.65×10^7
		27	24	9.28×10^6
		32	21	1.36×10^6
	LdMNPV	22	21	9.28×10^6
		27	21	5.22×10^6
		32	7	2.94×10^4
IPLB-HvE6a	AcMNPV	22	28	1.36×10^6
		27	28	9.28×10^5
		32	24	1.12×10^6
	HzSNPV	22	28	1.65×10^3
		27	28	2.00×10^3
		32	7	1.12×10^3

to lower temperatures than a pest of field crops. Alternatively, the *Helicoverpa/Heliothis* complex of insects typically occurs in warmer climates in such crops as cotton and would typically be exposed to much higher temperatures.

Conclusions

The results of these experiments show that low temperature maintenance of cells can provide a more accurate titration of nucleopolyhedroviruses, but at the cost of time. Previous studies have shown that titers can be obtained even more quickly through specific staining [11], but like plaque assays, their procedure requires fairly strong cell attachment. The advantage of the endpoint procedure described in this paper is that non-attached cell types can be utilized (and many insect cell lines do attach poorly or not at all) and that the procedure does not require materials beyond those typically used in cell culture laboratories.

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Notes on supplers

1. Lab-Line, Melrose Park, IL, USA.
2. Hotpack, Philadelphia, PA, USA, USA.
3. Clinical Scientific Equipment Company, Melrose Park, IL, USA
4. Corning, Corning, NY, USA.
5. JRH Biosciences, Lenexa, KS, USA.
6. Intergen, Purchase, NY, USA.
7. Life Technologies (GIBCO/BRL), Grand Island, NY, USA.

Mention of proprietary or brand names is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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